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Patterns of Protein Synthesis During the Second Cleavage of Mouse Two-Cell Embryos: Effects of Colcemid and α-Amanitin

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In this study, we attempted to determine the precise patterns of protein synthesis during the second cleavage in mouse. F1 hybrid 2-cell embryos showing a highly synchronized cell cycle and outbreed ICR strain 2-cell embryos were used. The patterns of protein synthesis during the second cleavage showed the sequential changes in the F1 hybrid and ICR strain 2-cell embryos. Moreover, we examined the effects of mitotic and transcriptional inhibitors such as colcemid and α-amanitin on the protein synthesis in the late 2-cell embryos of ICR strain. Treatment of colcemid (0.1 μg/ml) blocked the second cleavage, but did not affect on the change of protein synthesis. However, treatment of α-amanitin induced the synthesis of two set of polypeptides without affecting on synthesis of other proteins and cleavage. It thus seems that the appearance of α-amanitin-sensitive proteins may be not involved in the second cleavage.

Therefore, these results indicate that the second cell cycle in mouse embryos appears to be regulated at post transcriptional level, presumably independent on the expression of embryonic genome.

KEY WORDS: Mouse 2-cell embryo, Second cleavage, Protein synthesis, α-amanitin, Colcemid, F1 hydrid

Early development in mammalian embryos appears to take place largely or exclusively under the control of the maternal genome with the sequential activation and utilization of components synthesized and stored in the oocyte during oogenesis (Johnson, 1981; Pratt *et al.*, 1983). In the mouse embryo, the influence of maternal regulation appears to extend up to the early 2-cell stage. Although the limited synthesis of RNA in mouse 1-cell zygotes has been reported (Clegg and Piko, 1982, 1983), it is apparently not involved in regulation of the proximate development. Indeed, the physical enucleation or treatment with the transcriptional inhibitor such as α-amanitin, seem to not affect the normal sequence of morphological and molecular events that take place during development up to the early 2-cell stage (Braude *et al.*, 1979; Johnson, 1981; Schultz *et al.*, 1981; Van Blerkom, 1981). However, the sequential activation of subsets of mRNA and/or the post-translational modification of polypeptides appear to be involved in the early embryonic development (Cacio and Wassarman, 1982; Van Blerkom, 1981; Pratt *et al.*, 1983).

Several lines of investigation indicate that the transition from maternal to embryonic control of development in the mouse embryo may takes place in the 2-cell stage. Biochemical evidence

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suggests that the first major incorporation of RNA precusors into heterogeneous nuclear RNA (hnRNA) takes place during the 2-cell stage (Knowland and Graham, 1972; Clgg and Piko, 1982; Piko and Clegg, 1982). It coincides with major qualitative changes in profile of synthesis of polypeptides (Bolton *et al.*, 1984; Howlett and Bolton, 1985). The exact profile of protein synthesis during the second cell cycle is, however, not elucidated yet.

In this study, we attempted to determine the precise patterns of protein synthesis during the second cleavage in mouse. Moreover, we examined the effect of mitotic and transcription inhibitors such as colcemid and α-amanitin on the protein synthesis in the 2-cell embryos.

Materials and Methods

Collection and culture of embryos

ICR and F1 (C57BL/6 × CBA) hybrid strain female mice at 8-10 weeks of age were obtained from the Seoul National University Animal Breeding Center, and superovulated by injection of 5 i.u. pregnant mare's serum gonadotrophin (PMSG, Intervet) and followed by treatment of 5 i.u. human chorionic gonadotrophin (hCG, Intervet) 46 to 48 hr later. ICR females mated with males and a vaginal plug was checked in the following morning. Embryos were staged chronologically by designing the time of hCG injection as 0 hr. Mice were killed by cervical dislocation and the oviduct removed into medium 16 + 4 mg/ml of BSA (M16 + BSA; Whittingham, 1971). Early and late 2-cell embryos were obtained by flushing oviduct at 30 hr and at 48 hr, respectively. The blocked 2-cell embryos were obtained after culture of the early 2-cell embryos for 18 hr *in vitro*. Embryos were then culture in M16 + BSA under liquid paraffin oil in 5 % CO_2 in air at 37°C.

For fertilization *in vitro*, F1 hybrid males were killed, and the vas deference and cauda epididymis were dissected out and placed in a 1.0 ml of Whittingham's medium + 30 mg/ml of BSA (Frazer and Drury, 1975). The sperm were squeezed out gently and allowed to capacitate for 1.0 – 1.5 hr at 37°C. The females were killed 13.5 hr post hCG injection, and the oviducts were dissected out and the cumulus mass were released into a 0.5 ml of Whittingham's medium containing 30 mg/ml BSA. A 50 μl of the sperm suspension was then added to each 0.5 ml drop to give a final sperm concentration of approximately 1-2 × 10^6 sperm/ml. Eggs and sperm incubated together for 6 hr at 37°C at which time the eggs were transferred to M 16 + BSA and cultured.

[^{35}S]methionine labeling of 2-cell embryos

In vitro fertilized F1 hybrid 2-cell embryos were incubated with M16 + BSA containing 100 μ Ci/ml of [^{35}S]methionine (sp. act. approx. 1,100 Ci/mmole, New England Nuclear) for 2 to 4 hr at a different time during second cleavage. *In vivo* fertilized ICR 2-cell embryos were labeled with M16 + BSA supplemented 100 μCi/ml [^{35}S]methionine for 6 hr at a different time post hCG injection. Some of late 2-cell embryos were labeled with [^{35}S]methionine for 6 hr in the presence of 0.1 μg/ml of colcemid (Sigma) or 100 μg/ml of α-amanitin (Sigma) in order to examine the effects of meiotic and transcription inhibitors on the protein synthesis. After labeling, the embryos were thoroughly washed with Dubelcco's phosphate-buffered saline (PBS, pH 7.4), placed into 20 μl of SDS-sample buffer (Laemmli, 1970) and 30 μl of lysis buffer (O'Farrell, 1975). The sample was then stored at −70°C until use.

One dimensional SDS-polyacrylamide and two-dimensional gel electrophoresis

One dimensional SDS-PAGE was carried out by the method of Laemmli (1970) using a linear 8-15 % gradient separating gel and a 4 % stacking gel. For one-dimensional analysis, 20 embryos in each lane were used. Two-dimensional gel electrophoresis was performed according to the method described by O'Farrell (1975). The isoelectric focusing was carried out over a pH gradient of 4.5 to 7.0 using 2 % Ampholine (LKB) and the second dimension on a linear gradient 8-15 % polyacylamide containing 0.1 % SDS. One hundred embryos were used for 2-D gel electrophoretic analysis. After electrophoresis, the gels were fixed by immersing in 10 % acetic acid, and processed for fluorography as described by Bonner and Laskey (1974). The gels were then dried in vaccum at 80°C and exposed to X-ray

film (Kodak, X-Omat AR) under intensifying screen at −70°C for 1-4 weeks.

Results

Change of polypeptide synthesis during the second cleavage of 2-cell embryos fertilized *in vitro*

In order to obtain the precise profile of protein synthesis as function of time during the second cleavage, we used the F1 hybrid embryos which showed a highly synchronized cell cycle. *In vitro* fertilized 1-cell embryos of F1 hybrid were normally cleaved 19-21 hr post insemination (hpi) and sustained in state of 2-cell stage until 40-44 hpi. Thus, 2-cell embryos cleaved after fertilization *in vitro* were labeled with [^{35}S]methionine for 2- or 4-hr intervals during the second cell cycle and subjected to one-dimensional SDS-PAGE (Fig. 1) or 2-D gel electrophoresis (Fig. 2).

Fig. 1 depicts the sequential changes in protein synthesis during the second cell cycle. The polypeptides (large arrowheads in left. Fig. 1) synthesized mainly in the early 2-cell embryos are gradually decreased in their synthetic activity and nearly not detected at later stage. However, it appears that embryos at the late 2-cell stage may synthesize the new polypeptides (small arrowhead in light, Fig. 1). It is evident that the protein synthetic profile is changed as a function of insemination time as judged by two-D gel electrophoresis (Fig. 2).

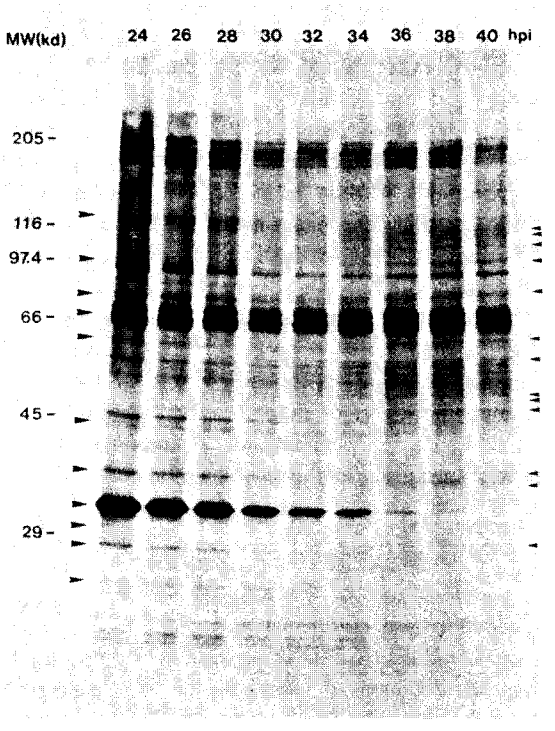

Fig. 1. One-dimensional SDS-PAGE showing the sequential protein synthesis pattern during the second cleavage of F1 hybrid 2-cell embryos fertilized *in vitro*. The 2-cell embryos obtained at different times post insemination (hpi) were labeled with [^{35}S]methionine for 2 hr and then subjected to SDS-PAGE. Large and small arrowheads indicate the polypeptides synthesized mainly in the early and later stage of 2-cell embryos, respectively. The numbers in left pannel represent the molecular weight markers.

Analysis of protein synthesis in 2-cell embryos fertilized *in vivo*

Based on the above *in vitro* results, we further analyzed the protein synthetic patterns in 2-cell embryos fertilized *in vivo* derived from ICR strain mouse. Two-cell mouse embryos were obtained from oviducts at different times post hCG and then labeled with [^{35}S] methionine for 4 hr. Fig. 3. shows that the pattern of protein synthesis is well consistent wth that observed in F1 hybrid 2-cell embryos (large arrowheads indicated the polypeptides synthesized mainely in the early 2-cell embryos, where small arrowheads indicated the new polypeptides in the late 2-cell embryos). Although the patterns of protein synthesis in the early and late 2-cell embryos remarkably differ, the pattern of protein synthesis in the blocked 2-cell embryos is very similar to the of the late 2-cell embryos except a minor difference (Fig. 4).

In the late 2-cell embryos in which cleavage is blocked by treatment of colcemid (0.1 μg/ml), but not affected by treatment of α-amanitin with a relatively high concentration (100 μg/ml) (Kidder *et al.*, 1985), the protein synthesis in both of late and locked 2-cell embryos is changes by α-amanitin but not by colcemid (Figs. 5 and 6). Notice that α-amanitin induced the synthesis of two set of polypeptides (shown by arrows in Figs. 5 and 6), which were synthesized at the early 2-cell embryos without affecting on the other protein in the late and block 2-cell embryos.

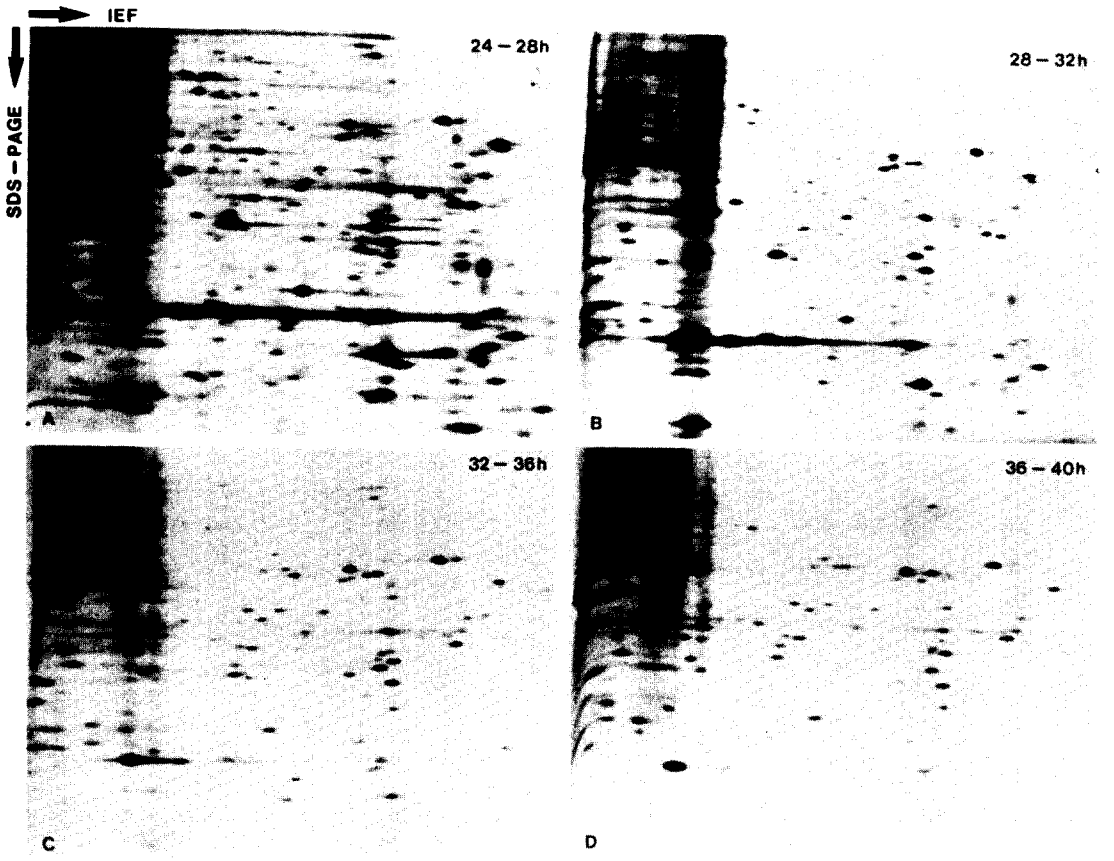

Fig. 2. Two-dimensional gel electrophoresis showing the sequential protein synthesis pattern during the second cleavage of F1 hybrid 2-cell embryos. The 2-cell embryos were labeled with [^{35}S]methionine for 4 hr at different times post insemination (hpi). A: 24-28 hpi, B: 28-32 hpi, C: 32-36 hpi, D: 46-40 hpi.

Disscusion

In the present study, we observed the precise pattern of protein synthesis during 2-cell embryo cleavage of the mouse development. A highly synchronized population of 2-cell embryos from F1 hybrid mouse fertilized *in vitro* or ICR strain 2-cell embryos fertilized *in vivo* were used. There are many reports dealing with the pattern of protein synthesis during the early embryonic development in mice, but most of them is mainly concerned on the first cleavage after fertilization. There are not many reports dealing with the protein synthesis during the second cleavage, in particular, of the outbreed strain such as ICR strain mice. It has been known that the second cell cycle of mouse embryos is relatively long (−20 hr), whereas the third and subsequent cycles are rather short (−12 hr) as the most conventional somatic cell cycle (Pratt, 1987). Thus, it is of importance to obtain embryos at particular stage either following hCG injection *in vivo* or after fertilization *in vitro*. However, it is always difficult since there is a substantial inter- and intra-embryo variation in developmental timing due to asynchrony of fertilization and/or the variation in cell cycle stage due to intrinsic, heritable differences in the blastomere (Bolton *et al.*, 1984; Pratt, 1987).

In order to overcome these difficulties to obtain a synchronized embryo, it is reasonable to use F1 hybrid embryos fertilized *in vitro* (Flach *et al.*, 1982; Howlett and Bolton, 1985) in F1 hybrid strain mouse such as C57BL × CBA F1 hybrid which does not exhibit *in vitro* 2-cell block (Pratt, 1987). As shown in Figs. 1 and 2, of po-

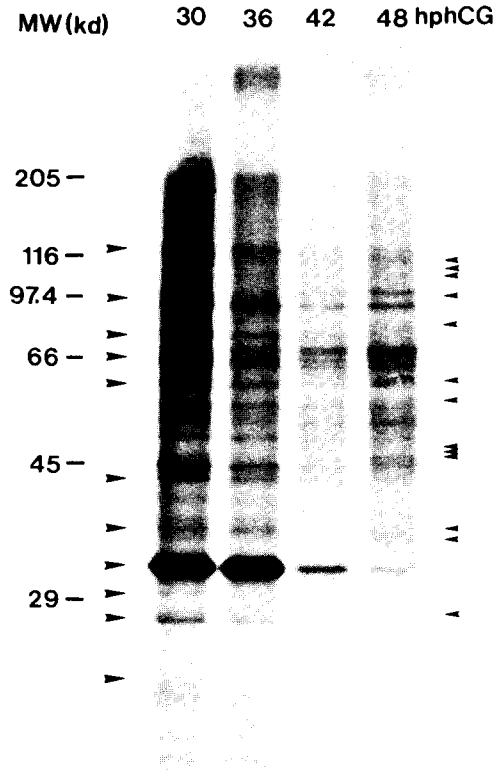

Fig. 3. One-dimensional SDS-PAGE showing the pattern of protein synthesis during the second cleavage in 2-cell embryos were obtained by flushing oviducts at different times post hCG and then labeled with [^{35}S]methionine for 6 hr. Anotation is the same as Fig. 1.

plypeptides synthesized mainly at the early 2-cell embryos, 67 kd polypeptide appears to be characteristic. This polypeptide is detected in 2-cell stage after the first cleavage (Flach *et al.*, 1982). Moreover, three other polypeptides such as 27, 35 and 46 kd proteins in the early 2-cell embryos of ICR strain as shown in Figs. 3 and 4 appear the major proteins synthesized from the late 1-cell to mid 2-cell embryo (Howlett and Bolton, 1985). Recently Howlett (1986) claimed that these proteins are M-phase components modified by protein phophorylation during the first cleavage and subsequent cell cycle.

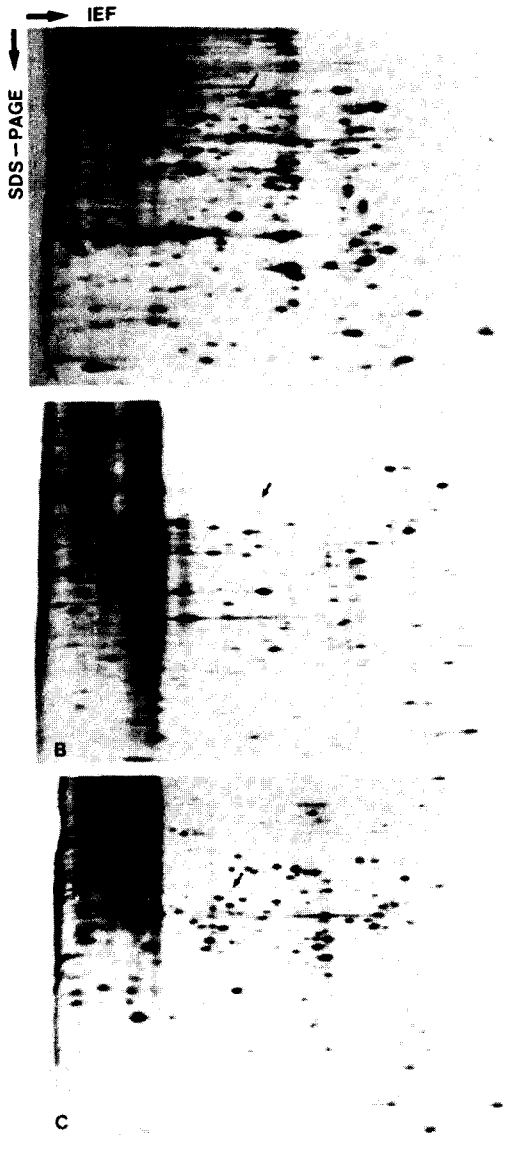

Fig. 4. Two-dimensional gel electrophoresis of the protein synthesis in 2-cell embryos of ICR strain. Early and late 2-cell embryos were obtained 30 and 46 hr post hCG respectiveley. The block 2-cell embryos were prepared from the culture of early 2-cell embryos for 18 hr *in vitro*. The embryos were labeled with [^{35}S] methionine for 6 hr. A: Early 2-cell embryos, B: Block 2-cell embryo, C: Late 2-cell embryo. Arrow indicates 67 kd protein as the representative early polypeptide.

It is of interest to mention that the pattern of protein synthesis of the blocked 2-cell embryos is

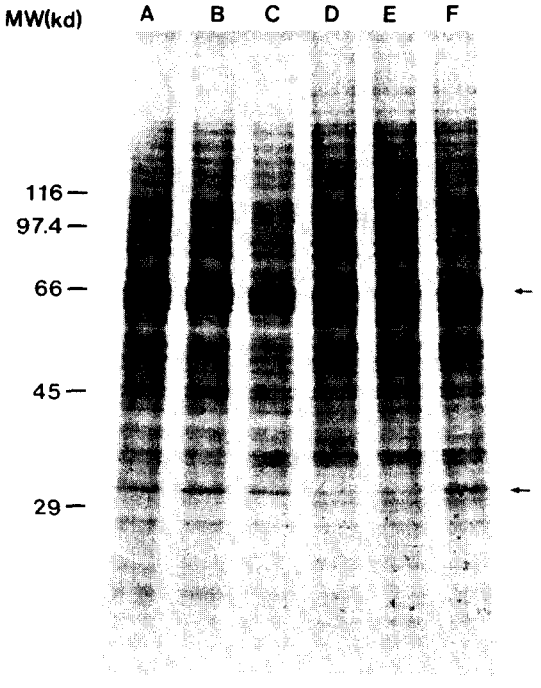

Fig. 5. Effect of treatment of colcemid and α-amanitin on the protein synthesis in the late and block 2-cell embryos. Late and block 2-cell embryos were prepared as described in Fig. 4 and labeled with [^{35}S]methionine for 6 hr in the presence of colcemid (0.1 μg/ml) and α-amanitin (100 μg/ml), respectively. Lane A: Block 2-cell embryos; Land B and C: Block 2-cell embryos treated with colcemid and α-amanitin, respectively; Lane D: Late 2-cell embryos; Lane E and F: Late 2-cell embryos treated with celcemid and α-amanitin, respectively. Arrows indicate the 35 and 67 kd polypeptides induced by α-amanitin.

just arrested at G_2 stage of cell cycle (Goddard and Pratt, 1983). Treatments of colcemid inhibited the cleavage, but did not produce any changes in protein synthesis. It appears then that treatment of colcemid is useful for the maintenance of mitotic phase of 2-cell embryos without any changes in protein synthesis.

It has been found that the treatment of α-amanitin to inhibit gene transcription is only sensitive just before and after S phase of the second cell cycle (Flach *et al.*, 1982). It appears to mark the first major expression of embryonic genome (Bolton *et al.*, 1984). Thus, the first detectable polypeptide synthetic events sensitive to α-amanitin occurred in two different phase at early and mid 2-cell stages (Flach *et al.*, 1982). The first α-amanitin sensitive events is completed soon after the first cleavage division, and results in the inhibition of synthesis of a complex of polypeptides with 67 kd representative in the early 2-cell embryo within few hours. These polypeptides are identified as a heat shock protein (Bensaude *et al.*, 1983). The second event occurs later in a mid-2-cell stage, and results in many changes in the polypeptide synthetic profile of 2-cell embryos. These events might represent the bursts of transcriptinal activity occurring prior to and immediatly after the second round of DNA replication. Thus it appears that the α-amanitin disrupts only the sequential utilization of maternal mRNA for stage-specific protein synthesis during the second cleavage (Bolton *et al.*, 1984). The present data, however, showed that treatment of α-amanitin at later stage induced only the synthesis of two set of polypeptides without inhibition of other protein synthesis and cytokinasis in both the late and blocked 2-cell embryos (Figs. 5 and 6). These polypeptides are synthesized mainly in the early 2-cell embryos and might be the same as a heat shock protein founded by Bensuade *et al.* (1983). It seems to reflect a stress effect of α-amanitin (Bensuade *et al.*, 1983) by using a relatively high cocentration of α-amanitin to completely inhibit mRNA synthesis in mouse 2-cell embryo (Kidder *et al.*, 1985). Therefore, it suggests that the second cell cycle in mouse embryo appears to be regulated at a post-transcriptional level, presumably independent on the expression of the embryonic genome, although embryonc genome is transcriptionaly active.

In summary, the mouse embryo of non-blocking strain such as F1 hybrid is likely to be useful for analysis of a precise pattern of protein synthesis involved in a specific deveopmental event during the second cleavage. And it seems that treatment of colcemid and α-amanitin in the late 2-cell embryos does not affect on the protein synthesis related to the second cleavage in mouse embryos.

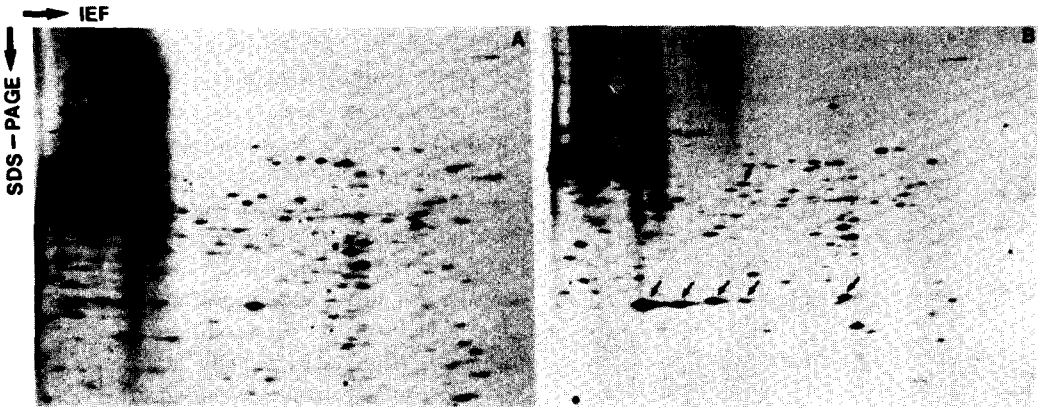

Fig. 6. Two-dimensional gel electrophoresis showing the effect of colcemid and α-amanitin on the protein synthesis in the late 2-cell embryos. The preparation and labeling of late 2-cell embryos is the same as the shown in Fig. 5. A: Colcemid; B: α-amanitin. Arrows indicate the 35 and 67 kd polypeptides induced by α-amanitin.

References

Bensaude, O., C. Babinet, M. Morange, and F. Jacob, 1983. Heat shock proteins, first major products of zygotic gene activity in the mouse embryo. *Nature* **305**:331-333.

Bonner, W. M. and R. A. Laskey, 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**:83-88.

Bolton, V. N., P. J. Oades, and M. H. Johnson, 1984. The relationship between cleavage, DNA replication, and gene expression in the mouse 2-cell embryo. *J. Embryol. Exp. Morphoe.* **79**:139-163.

Braude, P. R., H. R. B. Pelhan, G. Flach, and R. Lobatto, 1979. Post transcriptional control in early mouse embryo. *Nature* **282:102**-105.

Casio, S. M. and P. M‹. Wassarman, 1982. Program of early development in the mammals: Post transcriptional control of a class of proteins synthesized by mouse oocytes and early embryos. *Dev. Biol.* **89**:397-408.

Clegg, K. B. and L. Piko, 1982. RNA synthesis and cytoplasmic polyadenylation in the one-cell mouse embryo. *Nature* **292**:342-345.

Clegg, K. B. and L. Piko, 1983. Poly (A) length, cytoplasmic adenylation and synthesis of poly $(A)^+$ RNA in early mouse embryos. *Dev. Biol.* **95**:331-341.

Flach, G., M. H. Johnson, P. R. Braude, R. A. S. Taylor, anf V. N. bolton, 1982. The transition from maternal to embryonic control in the 2-cell mouse embryo. *EMBO J.* **1**:681-686.

Fraser, L. R. and L. Drury, 1975. The relationship between sperm concentration and fertilization *in vitro* of mouse eggs. Biol. Reprod. **13**:513-518.

Howlett, S. K., 1986. A set of proteins showing cell cycle dependent modification in the mouse embryo. *Cell* **45**:387-396.

Howlett, S. K. and V. N. Bolton, 1985. Sequence and regulation of morphological and molecular events during the first cell cycle of mouse embryogenesis. *J. Embryol. Exp. Morphoe.* **87**:175-206.

Johnson, M. H., 1981. The molecular and cellular basis of preimplantation mouse development. *Biol. Rev.* **56**:463-498.

Knowland, J. and C. F. Graham, 1972. RNA synthesis at the two-cell stage of mouse development. *J. Embryol. Exp. Morphoe.* **27**:167-176.

Kidder, G. M., A. F. Green, and J. R. McLachlin, 1985. On the use of α-amanitin as a transcriptional blocking agent in mouse embryo: A cautionary note. *J. Exp. Zool.* **233**:155-159.

Leammli, U. K., 1979. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**:680-685.

O'Farrell, P. H., 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**:4007-4021.

Piko, L. and K. B. Clegg, 1983. Quantitative changes in total RNA, total poly (A) and ribosomes in early mouse embryos. *Dev. Biol.* **89**:362-378.

Pratt, P. H. M., 1987. Isolation, culture and manupulation of preimplantation mouse embryos. *In* : Technique in Mammalian Development (Monk, M., ed.). IRL press, Practical approach Series, Oxford, pp. 13-42.

Pratt, H. P. M., V. N. Bolton, and K. A. Gudgeon, 1983. The legacy from the oocyte and its in controling early development of mouse embryo. *In* : Molecular Biolo-

gy of Egg Maturation, Ciba Symposium 98, Pitman Press, pp. 197-227.

Van Blerkom, J., 1981. Structural relationship and post translational modification of stage-specific proteins synthesized during early preimplantation development in the mouse. *Proc. Natl. Acad. Sci. USA* **78**:7629-7633.

Whittingham, D. G., 1971. cuture of mouse ova. *J. Reprod. Fert. (Suppl.)* **14**:447-478.

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생쥐 배아의 2세포기 분열과정에 있어서의 단백질 합성 분석 : Colcemid와 α-Amanitin의 영향

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본 연구는 생쥐 배아의 2세포기 분열과정중 단백질 합성양상과 단백질합성에 미치는 colcemid와 α-amanitin의 영향을 조사하였다. 이를 위하여 체내 수정된 ICR strain의 2세포기 배아와 매우 일치된 초기배아 분열양상을 보여주는 체외 수정된 F1 (C57BL × CBA) hybrid 2세포기 배아를 사용하였다. 두 종류의 2세포기 배아에서 단백질 합성은 분열단계에 따라서 매우 일치된 변화를 보여 주었다. 또한 유사분열 억제제인 colcemid (0.1 μg/ml)의 처리는 2세포기 배아분열을 억제하였으나, 단백질 합성에는 아무런 변화를 주지 못하였다. 그리고 후기 2세포기 배아에 전사 억제제인 α-amanitin (100 μg/ml)을 처리하였을 때 세포분열이나 다른 단백질의 합성에는 아무런 영향이 없이 단지 두개의 단백질의 합성만을 유도하였다. 이는 아마도 α-amanitin의 stress효과에 기인하는 것으로 추측된다.

따라서 생쥐 2세포기 배아의 분열과정은 배아게놈의 유전자 발현과는 무관하게 이미 합성되어 존재하는 mRNA에 의하여 조절되는 것으로 사료된다.